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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [5] :		(11) International Publication Number:	WO 93/14200
C12N 15/00, A01K 67/027 C12N 5/10, C07K 15/00	A1	(43) International Publication Date:	22 July 1993 (22.07.93)

(21) International Application Number: PCT/US92/11276

(22) International Filing Date: 29 December 1992 (29.12.92)

(30) Priority data:

817,584	7 January 1992 (07.01.92)	US
915,469	16 July 1992 (16.07.92)	US

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(81) Designated States: AU, CA, FI, JP, NO, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: TRANSGENIC ANIMAL MODELS FOR ALZHEIMER'S DISEASE

(57) Abstract

The construction of transgenic animal models for testing potential treatments for Alzheimer's disease are described. The models are characterized by a greater similarity to the conditions existing in naturally occurring Alzheimer's disease, based on expression of all three forms of the β-amyloid precursor protein (APP), APP_{695}, APP_{751}, and APP_{770}, as well as various point mutations based on naturally occurring mutations, such as the London and Indiana familial Alzheimer's disease (FAD) mutations at amino acid 717, and predicted mutations in the APP gene. The APP gene constructs are prepared using the naturally occurring promoter, as well as inducible promoters such as the mouse metallothionine promoter, which can be regulated by addition of heavy metals such as zinc to the animal's water or diet, and promoters such as the rat neuron specific enolase promoter, human β actin gene promoter, human platelet derived growth factor B (PDGF-B) chain gene promoter, rat sodium channel gene promoter, mouse myelin basic protein gene promoter, human copper-zinc superoxide dismutase gene promoter, and mammalian POU-domain regulatory gene promoter. The constructs are introduced into animal embryos using standard techniques such as microinjection. Animal cells can be isolated from the transgenic animals or prepared using the same contructs with standard techniques such as lipofection or electroporation. The transgenic animals, or animal cells, are used to screen for compounds altering the pathological course of Alzheimer's disease as measured by their effect on the amount and histopathology of APP and β-amyloid peptide in the animals, as well as by behavioral alterations.

Transgenic An nal Models for Alzheimer's Disease

Bac ground of the Invention

Transgenic t chnology is described for the production of ani als that exhibit symptoms of human Alzheimer's dise e through the expression of the Alzheimer's precu sor protein or a modified version thereof.

Alzheimer's isease (AD) is a degenerative disorder of the ain first described by Alios Alzheimer in 190 after examining one of his patients who suffered dra ic reduction in cognitive abilities and had generali d dementia ("The early story of Alzheimer's Dise e", edited by Bick K., Amaducci L., and Pepeu G. (Ra n Press, New York 1987). It is the leading cause of lementia in elderly persons. AD patients have in eased problems with memory loss and intellectual func ions which progress to the point where they canno function as normal individuals. With the loss of intellectual skills the patients exhibit personal y changes, socially inappropriate actions and schi phrenia ("A guide to the understanding of lzheimer's Disease and related disorders", edit by Jorm AF.; (New York University Press, New York 1 87). AD is devastating for both victims and thei families, for there is no effective palliative or pr entive treatment for the inevitable neurodegeneratio The most common problems in the Alzheimer's pati t are inability to dress unaided, restlessness by c y, urinary incontinence and sleep disturbances. T family members report embarrassment, a iety, depression, and a decreased social life.

The impact AD on society and on the national economy is enorm s. It is expected that the demented elderly populati in the United States will increase by 41% by the ye 2000. It is expensive for the health care syst s that must provide institutional and ancillary ca for the patients at an estimated

neuropathy. Howe er, available evidence strongly indicates two dis inct types of genetic predisposition for AD. First, m lecular analysis has provided evidence for muta ions in the amyloid precursor protein (APP) gen in certain AD-stricken families (Goate, et al. Na ure 349:704-706 (1991); Murrell, J, et al. Science 25 ; 97-99, 1991; Chartier-Harlin, M-C, et al. Nature 353 844-846 (1991)). Second, in certain other fam lies with a clear genetic predisposition to AD, the mutation maps to chromosome 21 but is distinc from the APP locus (Tanzi, R.E., et al. Nature, 331;5 3-530 (1988)).

Amyloid plag es are abundantly present in AD patients and in D wn's Syndrome individuals surviving to the age of 40. The plaques are also present in the normal aging brai , although at a lower number. These plaques are made p of the amyloid ß peptide (ß peptide) (Glenner and Wong, et al., Biochem. Biophys. Res. Comm. 120:88 -890 (1984)), which is also the main protein constitue t in cerebrovascular deposits and neurofibrillary t ngles. The β peptide is a filamentous mater al that is arranged in beta-pleated sheets and has a olecular weight of 4.2-4.5 kd. It is a hydrophobic eptide comprising 39-42 amino acids. The determination of its amino acid sequence led to the cloning of th APP cDNA (Kang, et al., Nature 325:733-735 (1987 ; Goldgaber, et al., Science 235:877-880 (1987 ; Robakis et al., Proc. Natl. Acad. Sci. 84:4190-4194 (1987); Tanzi, et al., Nature 331:528-530 (1988 and genomic APP DNA (Lemaire et al., Nucl. Acids s. 17:517-522 (1989); Yoshikai, et al., Gene 87, 257 263 (1990). Three forms of APP cDNAs (APP695, AP 751, and APP770) have been isolated, and arise from a ingle precursor RNA by alternate splicing. The ge spans more than 175 Kb with 18 exons (Yoshikai, al., 1990). APP contains three extracellular dom ins, a transmembrane region and a

of the full leng[illegible] protein (Goate, et al., (1991); Murrell et al., [illegible]91; Chartier-Harlin et al., 1991). These mutations [illegible]-segregate with the disease within the families and [illegible]re absent in families with late-onset AD.

There are n[illegible] proven animal models to study AD, although aging n[illegible] human primates seem to develop amyloid plaques [illegible] ß peptide in brain parenchyma and in the walls of [illegible]me meningeal and cortical vessels. Although aged pri[illegible]ates and canines can serve as animal models, they are [illegible]xpensive to maintain and need lengthy study pe[illegible]ods. There are no spontaneous animal mutations [illegible]ith sufficient similarities to AD to be useful as exp[illegible]imental models. Various models have been proposed in [illegible]hich some AD-like symptoms may be induced by elect[illegible]lysis, transplantation of AD brain samples, aluminu[illegible] chloride, kainic acid or choline analogs (Kisner, [illegible]t al., Neurobiol. Aging 7;287-292 (1986); Mistry, [illegible] S., et al., J Med Chem 29;337-343 (1986)). Flood, [illegible]t al. (Proc. Natl. Acad. Sci. 88:3363-3366 (19[illegible]), reported amnestic effects in mice of four synthetic peptides homologous to the ß peptide. Because none of these share with AD either common symptoms, [illegible]iochemistry or pathogenesis, they are not likely to yield much useful information on etiology or treat[illegible]ent.

Transgenic [illegible]ce with the human APP promoter linked to *E. coli* ß-galactosidase (Wirak, D.O., et al., The EMBO J [illegible];289-296 (1991)) as well as transgenic mice [illegible]pressing the human APP751 cDNA (Quon, D, et al. [illegible]ature 352, 239-241 (1991)) or subfragment of th[illegible] cDNA including the ß peptide (Wirak, D.O., et [illegible]l., Science 253, 323-325 (1991); Sandhu, F.A., et [illegible]l., J. Biol. Chem. 266, 21331-21334 (1991); Kawabata, [illegible]S. Nature 354, 476-478 (1991)) have been produced. [illegible]sults obtained in the different studies appear to depend upon the source of promoter

Alzheimer's isease is a complex syndrome involving patholc ical and behavioral aspects. A useful disease mc el should take these complexities into account. Th re are multiple proteins expressed from the gene wit certain forms predominating in a given tissue. In the brain, the 695 form is predominant, but he mRNAs for additional forms are also present (Gol e et al., Neuron 4; 253-267 (1990)). It is not known w ether the ratio of the different forms changes wit the age of the individual. The various protein f rms result from alternative splicing such that the KI omain and/or the OX-2 domain may or may not be preser in the mature protein. Moreover, the ß-peptide res lts from post-translational processing of the precursor protein. This process can change in time as an individual ages, and can be affected by mutat ons not directly affecting the structure of the -peptide:for example, the familial Alzheimer's disea e (FAD) mutations at amino acid position 717 in t e full length protein (Groate, et al., 1991; Murrel , et al., 1991; Chartier-Harlin, et al., 1991). Give these considerations, the production of uni ersal animal models for Alzheimer's disease necessita es the construction of animal models that take into ac ount the effects of known mutations on the phenotype esulting from the expression of these forms, and he possibility of the ratio of the different forms c anging during the lifetime of the animal.

It is theref re an object of the present invention to prov de an animal model for Alzheimer's disease that is c nstructed using transgenic technology.

It is a furt er object of the present invention to provide transg nic animals that accurately reflect the expression of different forms of the amyloid precursor protein

construct; the AI 751 cDNA and bearing a mutation at
amino acid 717; t e APP695 cDNA; the APP695 cDNA
bearing a mutatic at amino acid 717; the APP leader
sequence followed by the ß peptide region plus the
remaining carboxy terminal 56 amino acids of APP; the
APP leader sequer e followed by the ß peptide region
plus the remainin carboxy terminal 56 amino acids
with the additior of a mutation at amino acid 717; the
APP leader sequer e followed by the ß peptide region;
the ß peptide reg on plus the remaining carboxy
terminal 56 amino acids of APP; the ß peptide region
plus the remainir carboxy terminal 56 amino acids of
APP with the addi ion of a mutation at amino acid 717;
a combination ger mic-cDNA APP gene construct; and a
combination genor c-cDNA APP gene construct, with the
addition of a mut tion at amino acid 717, operably
linked to promot s selected from the following: the
human APP gene pr moter, mouse APP gene promoter, rat
APP gene promoter metallothionine gene promoter, rat
neuron specific e olase gene promoter, human ß actin
gene promoter, hu an platelet derived growth factor B
(PDGF-B) chain ge e promoter, rat sodium channel gene
promoter, mouse m elin basic protein gene promoter,
human copper-zinc superoxide dismutase gene promoter,
and mammalian POU domain regulatory gene promoter.
Additional constr cts include a human yeast artificial
chromosome constr ct controlled by the human APP
promoter; a human yeast artificial chromosome
construct control ed by the human APP promoter with
the addition of a mutation at amino acid 717; the
endogenous mouse r rat APP gene modified through the
process of homolo ous recombination between the APP
gene in a mouse e rat embryonic stem (ES) cell and a
vector carrying t e human APP cDNA of the wild-type
such that sequen s in the resident rodent chromosomal
APP gene beyond t e recombination point (the preferred
site for recombir tion is within APP exon 9) are

Figure 2b is a schematic of the APP751 cDNA coding sequence bearing a mutation at position 717.

Figure 3a is a schematic of the APP695 coding sequence.

Figure 3b is a schematic of the APP695 cDNA coding sequence bearing a mutation at position 717.

Figure 4a is a schematic of a coding sequence for the carboxy terminal portion of APP.

Figure 4b is a schematic of a coding sequence for the carboxy terminal portion of APP bearing a mutation at position 717.

Figure 5 is a schematic of a coding sequence for the ß peptide portion of APP.

Figure 6a is a schematic of a combination genomic/cDNA coding sequence allowing alternative splicing of the KI and OX2 exons.

Figure 6b is a schematic of a combination genomic/cDNA coding sequence bearing a mutation at position 717 and allowing alternative splicing of the KI and OX2 exons.

Figure 7a is a schematic of a human APP YAC coding sequence.

Figure 7b is a schematic of a human APP YAC coding sequence bearing a mutation at position 717.

Figure 8 is a schematic of genetic alteration of the mouse APP gene by homologous recombination between the mouse APP gene in a mouse ES cell and a vector carrying the human APP cDNA (either of the wild-type or FAD mutant form) directed to the exon 9 portion of the gene. As a result of this recombination event, sequences in the resident mouse chromosomal APP gene beyond the recombination point in exon 9 are replaced by the analogous human sequences.

Isolation of the uman APP promoter.

A cosmid li ary, constructed from human placental DNA in he pWE15 cosmid vector, was screened by hybridization ith a ^{32}P-labeled probe prepared by nick-translation Maniatis, et al. Molecular Cloning: a laboratory man l (Cold Spring Harbor Laboratory, Cold Spring Harb , NY 1989)) of the APP770 cDNA clone. Clones t t hybridized with the probe were picked, purified, and characterized by restriction mapping, hybridi tion, and DNA sequencing. From one such clone conta ing a long 5′ flanking region, a *Not*I to *Nru*I res iction DNA fragment of approximately 25 kb was isolat . This fragment terminates 2 nucleotides befo the initiator methionine codon of the Alzheimer's otein-coding region. This fragment, or a subfragment hereof, is the source of the human APP promoter for he constructs described herein. Analogous DNA fr ments isolated using the same methods from mou or rat genomic libraries are the source of mouse rat promoters.

Definition of AP cDNA clones.

The cDNA cl e APP-695 is of the form of cDNA described by Kan et al., Nature 325:733-735 ((1987), and represents t most predominant form of Alzheimer's prot n in the brain. The cDNA clone APP-751 is of the fo described by Ponte, P, Nature 331, 525-527 (1988). he cDNA clone APP-770 is of the form described by Kit uchi, et al. Nature 331:530-532 (1988). This fo contains an insert of 225 nucleotides rela ve to the 695 form. The 225 nucleotide inser encodes for the KI domain as well as the OX-2 domain.

Definition of th APP genomic locus.

Characteriz ion of phage and cosmid clones of human genomic DN clones listed in the table below originally estab shed a minimum size of at least 100 kb for the Alzhe er's gene. There are a total of 18

addition to thos indicated above. First, an APP770 cDNA clone is di sted with Asp718 which cleaves after position 56 (num ring system of Kang et al., 1987). The resulting 5′ xtension is filled in using the Klenow enzyme (M iatis et al., 1989) and ligated to a hexanucleotide o the following sequence: AGATCT, the recognition site or *Bgl*II. After cleavage with *Bgl*II, which als cuts after position 1769, and re-ligation, the tr slational reading frame of the protein is prese ed. The truncated protein thus encoded contains he leader sequence, followed by approximately 6 ino acids that precede the ß peptide, followe by the ß peptide, and the 56 terminal amino a ds of APP. The clone in Fig. 5 is created by the i roduction through site directed mutagenesis of n leotide 1913 in the clone of Fig. 4a (numbering syste of Kang et al., 1987) to a T thus creating a termi tion codon directly following the last amino acid c don of the *β* peptide. Each of the APP cDNA sequenc clones shown in Figs. 1-5 contains a single *Nru*I site nucleotides upstream from the initiator methio ne codon that is used for attachment of the different romoters used to complete each construct.

Expression c ones identical to these but bearing mutations at the mino acid 717 of the full length protein, the sit of the FAD mutation, are also constructed. Mu tions at amino acid 717 are created by site-directed utagenesis (Vincent, et al., Genes & Devel. 3, 334-34 (1989)) and include mutations of the wild-type val co n to one of the following codons; ile, phe, gly, t , leu, ala, pro, trp, met, ser, thr, asn, gln.

The preferr method for construction of the combination cDNA/ enomic expression clones in Figure 6 is as follows. e *Taq*I site at position 860 (numbering syste of Kang, et al., 1987) in an APP770

Activity of ene Promoters.

Different p moter sequences are used to control expression of AP coding sequences. The ability to regulate expressi n of the APP gene in transgenic animals is belie d to be useful in evaluating the roles of the dif rent APP gene products in AD. The ability to regul e expression of the APP gene in cultured cells i believed to be useful in evaluating expression and p cessing of the different APP gene products and may ovide the basis for cell cultured drug screens.

The metallot ionine (MT) promoter is well characterized, ha been employed in transgenic animals, and its xpression can be regulated through modulation of zi and glucocorticoid hormone levels (Palmiter et al., Nature 300, 611-615 (1982)).

The human AP promoter is also characterized with regard to expres on in the CNS (Wirak et al., 1991). It is believed th this promoter is useful for accurately reprod cing temporal and spatial expression of human APP sequ nces in the CNS of transgenic rodents. In addi ion to the human APP promoter, the APP promoter fro mouse and rat is used in conjunction with the various ild-type and mutant APP coding sequences. Altho gh the human APP promoter has been shown to have act vity in the appropriate regions of the brain of tran genic mice (Wirak et al., 1991), it is believed that e use of a mouse APP promoter in a transgenic mouse a rat APP promoter in a transgenic rat will offer an even more precise pattern of expression in the NS of transgenic animals.

As an altern ive for the control of human APP expression in neu ns, the rat neuron specific enolase gene promoter is ed. This promoter has been shown to direct express n of coding sequences in neurons (Forss-Petter et ., Neuron 5;197-197 (1990)).

The YAC-APP lone, shown in Figu e 7a, is established in en ryonic stem (ES) ce ls by selecting for neomycin resi tance encoded by the YAC vector. ES cells bearing the YAC-APP clone are used to produce transgenic mice b established method described below under "Transgenic Mice" and "Embryoni Stem Cell Methods". The YA -APP gene bearing a mutation at amino acid 717 (F g. 7b) is produced through the generation of a Y C library using genomic DNA from a person affected b a mutation at amino acid 717. The clone is identifi d and established in ES cells as described above.

Genetic Alteratio of the Mouse APP G ne.

The nucleoti e sequence homology between the human and murine lzheimer's protein genes is approximately 85% Within the β peptide-coding region, there are hree amino acid differences between the two sequences The val residue t at is mutated at amino acid 717 is conserved between m use, rat, and man. Wild-type r dents do not develo Alzheimer's disease nor do th y develop deposits or plaques in their CNS analogo s to those present i human Alzheimer's patie ts. Therefore, it i possible that the human but not the rodent form of ß eptide is capable of causir disease. Homologous recombination (Capecchi, MR Sci nce 244, 1288-1292 989)) can be used to convert t e mouse Alzheimer's gene *in situ* to a gene encoding t e human ß peptide. This recombination is irected to a site do nstream from the KI and OX-2 d mains, for example, within exon 9, so that the natur l alternative splicing mechanisms appropriate to al cells within the t nsgenic animal can be employed i expressing the fin l gene product.

Both wild-ty e (Fig. 8, schematic "a") and mutant (Fig. 8, schemati "b") forms of human cDNA are used to produce transg nic models expressin either the wild-type or muta e forms of APP. The recombination

is redissolved in ml of low salt buffer (0.2 M NaCl, 20 mM Tris™, pH 7.4, and 1 mM EDTA) and purified on an Elutip-D™ column. The column is first rinsed with 3 ml of high salt buffer (1 M NaCl, 20 mM Tris™, pH 7.4, and 1 mM EDTA) followed by washing with 5 ml of low salt buffer. The DNA solutions are passed through the column for three times to bind DNA to the column matrix. After one wash with 3 ml of low salt buffer, the DNA is eluted with 0.4 ml of high salt buffer and precipitated by two volumes of ethanol. DNA concentrations are measured by absorption at 260 nm in a UV spectrophotometer. For microinjection, DNA concentrations are adjusted to 3 µg/ml in 5 mM Tris™, pH 7.4 and 0.1 mM EDTA. Other methods for purification of DNA for microinjection are also described in Hogan et al., Manipulating the mouse embryo (cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1986); in Palmiter, et al., Nature 300, 611 (1982), in "The Qiagenologist, Application Protocols", 3rd edition, published by Qiagen, Inc., Chatsworth, CA., and in Maniatis, et al. Molecular Cloning: a laboratory manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 1

Construction of Transgenic Animals.

Animal Source

Animals suitable for transgenic experiments can be obtained from standard commercial sources such as Charles River (Wilmington, MA), Taconic Farms (Germantown, NY), Harlan Sprague Dawley (Indianapolis, IN), etc. Swiss Webster female mice are preferred for embryo retrieval and transfer. B6D2F$_1$ males can be used for mating and vasectomized Swiss Webster studs can be used to stimulate pseudopregnancy. Vasectomized mice and rats can be obtained from the supplier.

Microinjection Procedures

The procedure for manipulation of the rodent embryo and for microinjection of DNA are described in

transferred. Aft the transfer, th inc sion is closed by two sut es.

Transgenic R s

The procedur for generating t. nsgeric rats is similar to that o mice (Hammer et ., Cell 63;1099-112 (1990)). Thi y day-old female ts are given a subcutaneous inje ion of 20 IU of G (.1 cc) and 48 hours later ea female placed wit a proven male. At the same time, 0-80 day old fem s are placed in cages with vasect ized males. The will provide the foster mothers fo embryo transfer. The next morning females are check for vaginal plu . Females who have mated with v ectomized males held aside until the time of ransfer. Donor ale that have mated are sacrifi d (CO_2 asphyxiation and their oviducts removed, placed in DPBS (D ecco's phosphate buffered saline) th 0.5% BSA and embryos collected. Cumul cells surroundi the embryos are removed with hyal onidase (1 mg/ml Th embryos are then washed and p ced in EBSS (Ear s balanced salt solution) contain g 0.5% BSA in a 37.5°C incubator until the time of icroinjection.

Once the emb os are injected, e live embryos are moved to DPBS or transfer into ster mothers. The foster mother are anesthetized ith Ketamine (40 mg/kg, ip) and xy zine (5 mg/kg, ip . A dorsal midline incision is made through th ski and the ovary and oviduct re exposed by an cision through the muscle layer directly over the y. The ovarian bursa is torn, th embryos are pick up into the transfer pipet, a the tip of the ransfer pipet is inserted into the infundibulum. Ap roxim tely 10-12 embryos are trans rred into each r oviduct through the infundibulum. The incision is losed with sutures, and the ster mothers are used singly.

DNA molecule introduced into ES cells can also be integrated into the chromosome through the process of homologous recombination, described by Capecchi, (1989). Direct injection results in high efficiency of integration. Desired clones are identified through PCR of DNA prepared from pools of injected ES cells. Positive cells within the pools are identified by PCR subsequent to cell cloning (Zimmer and Gruss, Nature 338, 150-153 (1989). DNA introduction by electroporation is less efficient and requires a selection step. Methods for positive selection of the recombination event (i.e., neo resistance) and dual positive-negative selection (i.e., neo resistance and gancyclovir resistance) and the subsequent identification of the desired clones by PCR have been described by Joyner et al., Nature 338, 153-156 (1989) and Capecchi, (1989), the teachings of which are incorporated herein.

Embryo recovery and ES cell Injection

Naturally cycling or superovulated female mice mated with males are used to harvest embryos for the implantation of ES cells. It is desirable to use the C57B strain for this purpose when using mice. Embryos of the appropriate age are recovered approximately 3.5 days after successful mating. Mated females are sacrificed by CO_2 asphyxiation or cervical dislocation and embryos are flushed from excised uterine horns and placed in Dulbecco's modified essential medium plus 10% calf serum for injection with ES cells. Approximately 10-30 ES cells are injected into blastocysts using a glass microneedle with an internal diameter of approximately 20 μm.

Transfer of Embryos to Pseudopregnant Females

Randomly cycling adult female mice are paired with vasectomized males. Mouse strains such as Swiss Webster, ICR or others can be used for this purpose.

observed in deeper grey matter including the amygdaloid nucleus corpus striatum and diencephalon. Sections are also stained with other antibodies diagnostic of Alzheimer's plaques, recognizing antigens such as Alz-50, tau, A2B5, neurofilaments, neuron-specific enolase, and others that are characteristic of Alzheimer's plaques (Wolozin, et al., Science 232, 648 (1986); Hardy and Allsop, Trends in Pharm. Sci. 12, 383-388 (1991); Selkoe, Ann. Rev. Neurosci. 12, 463-490 (1989); Arai et al., Proc. Natl. Acad. Sci. USA 87, 2249-2253 (1990); Majocha et al., Amer. Assoc. Neuropathology Abs; 99,22 (1988); Masters et al., Proc. Natl Acad. Sci. 82,4245-4249; Majocha et al., Can J Biochem Cell Biol 63;577-5 (1985)). Staining with thioflavins and congo red is also carried out to analyze co-localization of ß peptide deposits within neuritic plaques and NFT.

Analysis of APP and ß Peptide Expression:

mRNA: mRNA is isolated by the acid guanidinium thiocyanate-phenol chloroform extraction method (Chomczynski and Sacchi, Anal Biochem 162:156-159 (1987)) from cell lines and tissues of transgenic animals to determine expression levels by Northern blots.

Protein: APP and ß peptide are detected by using polyclonal and monoclonal antibodies that are specific to the extra-cytoplasmic domain, ß peptide region, and C-terminus.

Western Blot Analysis: Protein fractions are isolated from tissue homogenates and cell lysates and subjected to Western blot analysis as described by Harlow et al., Antibodies: A laboratory Manual, (Cold Spring Harbor, NY, 1988); Brown et al., J Neurochem. 40;299-308 (1983); and Tate-Ostroff et al., Proc Natl Acad Sci 86;745-74 (1989)). Only a brief description is given below.

are detected by autoradiography or enzymatically labeled probes are detected through reaction with the appropriate chromogenic substrates.

Behavioral Studies

Behavioral tests designed to assess learning and memory deficits are employed. An example of such a test is the Morris Water maze (Morris, J. Motivat. 12;239-260 (1981)). In this procedure, animal is placed in a circular pool filled with water with an escape platform submerged just below the surface of the water. A visible marker is placed on the platform so that the animal can find it by navigating toward a proximal visual cue. Alternatively, a more complex form of the test in which there are no distal cues to mark the platform's location will be given the animals. In this form, the animal must learn the platform's location relative to distal visual cues.

The procedure as applied to test transgenic mice is similar for transgenic rats.

Screening of Compounds for Treatment of Alzheimer's Disease

The transgenic animals and animal cells are used to screen compounds for a potential effect in the treatment of Alzheimer's disease using standard methodology. The compound is administered to the animals or introduced into the culture medium over a period of time and in various dosages, the animals or animal cells examined for alterations in APP expression, histopathology, and/or behavior using the procedures described above.

Example 1: Expression of pMTAPP-1 in NIH3T3 and PC12 Cells.

The clone, pMTAPP-1 is an example of an expression vector shown in Fig. 1a where the promoter used is the metallothionine promoter. Stable cell lines were derived by transfecting NIH3T3 and PC12 cell lines (ATCC #CCL92 and CRL1721). 0.5×10^{-6} of

human APP770 cDNA like the construct in re 1A. DNA from this construct was transfected PC12 cells as described above. Certain clones EAPP-1 transfected cells exhibited a differenti n phenotype morphologically similar to tha hibited by PC12 cells treated with nerve growth fac (NGF). PC12 cells normally are fairly round and t cells. Those transfected with pEAPP-1 have cyto mic extensions resembling neurites. PC12 cel treated with NGF extend very long neuritic extens s. Thirteen PC12 cell clones transfected wi EAPP-1 were selected and propagated. Eight of e cell clones exhibited the spontaneous differe tion phenotype with clones 1-8, 1-1, and 1-4 biting the strongest phenotypes. Staining of pEAPP nsfected PC12 cells with antibody against the ß pe de as described above indicated that those cel xhibiting the differentiation were also expressing . Because PC12 cells transfected with the pMTAPP1 e did not exhibit this phenotype even though the A 0 cDNA is expressed, these results suggest tha ex sion of APP770 from the human promoter has novel perties regarding the physiology of the cell.

Example 3: Expression of pMTA4 in PC ells.

pMTA4 is an example of the type of c truct shown in Figure 4A where the promoter use s the metallothionine promoter. The protein er ed by this construct differs slightly from tha dep d in Figure 4. An APP770 cDNA clone was ges with Asp718 which cleaves after position 6 (r system of Kang, et al., 1987). The resulting 5 tension was filled in using the Klenow enzyme (Ma tis). The same DNA preparation was also cleaved wit coRI which also cuts after positi n 1795 and th res ng 5' extension was filled in using the Kl row e (Maniatis). Self-ligation of this molec sults in an expression clone in which the truncat otein

a humidified atmosphere at 7% CO_2, 5% O_2, and 88% N_2 until the time of injection.

Microinjection: Elutip-D™ purified Sal I DNA was dissolved in 5 mM Tris (pH 7.4) and 0.1 mM EDTA at 3 μg/ml concentration for microinjection. Microneedles and holding pipettes were pulled from Fisher coagulation tubes (Fisher) on a DKI model 720 pipette puller. Holding pipettes were then broken at approximately 70 μm (O.D.) and fire polished to an I.D. of about 30 μm on a Narishige microforge (model MF-83). Pipettes were mounted on Narishig micromanipulators which were attached to a Nikon Diaphot microscope. The air-filled injection pipette was filled with DNA solution through the tip after breaking the tip against the holding pipette. Embryos, in groups of 30 to 40, were placed in 100 μl drops of EBBS under paraffin oil for micromanipulation. An embryo was oriented and held with the holding pipette. The injection pipette was then inserted into the male pronucleus (usually the larger one). If the pipette did not break through the membrane immediately the stage was tapped to assist in penetration. The nucleus was then injected and the injection was monitored by swelling of the nucleus. Following injection, the group of embryos was placed in EBSS until transfer to recipient females.

Transfer: Randomly cycling adult female mice were paired with vasectomized Swiss Webster males. Recipient females were mated at the same time as donor females. At the time of transfer, the females were anesthetized with avertin. The oviducts were exposed by a single midline dorsal incision. An incision was then made through the body wall directly over the oviduct. The ovarian bursa was then torn with watch makers forceps. Embryos to be transferred were placed in DPBS and in the tip of a transfer pipet (about 10-12 embryos). The pipet tip was inserted into the

We claim:

1. A non-human transgenic mammal or mammalian cells containing a construct selected from the group consisting of the APP770 cDNA; the APP77[illegible] DNA bearing a mutation at amino acid 717; the APP751 [illegible]A containing the KI protease inhibitor doma[illegible] without the OX2 domain in the construct; the APP75[illegible] cDNA bearing a mutation at amino acid 717; the [illegible]PP695 cDNA; the APP695 cDNA bearing a mutation at am[illegible] acid 717; the APP leader sequence followed by the [illegible] peptide region plus the remaining carboxy termin[illegible]6 amino acids of APP; the APP leader sequence fo[illegible]ed by the ß peptide region plus the remaining carbo[illegible] terminal 56 amino acids with the addition of a mut[illegible]on at amino acid 717; APP leader sequence follo[illegible] by the ß peptide region; the ß peptide region plu[illegible] remaining carboxy terminal 56 amino acid[illegible]PP; the ß peptide region plus the remaining carb[illegible] terminal 56 amino acids of APP with the addition [illegible]utation at amino acid 717; a combination genomic-[illegible] APP gene construct; a combination genomic-cDNA API[illegible]e construct, with the addition of a mutati[illegible] amino acid 717, operably linked to a promoter [illegible]ed from the following: the human APP promoter, r[illegible]PP promoter, rat APP promoter, metallothion[illegible]promoter, rat neuron specific enolase promoter, hur[illegible] actin gene promoter, human platelet derived gr[illegible] factor B (PDGF-B) chain gene promoter, rat sodium [illegible]nel gene promoter, mouse myelin basic protein gen[illegible]noter, human copper-zinc superoxide dismutase [illegible]promoter, and mammalian POU-domain regulatory gen[illegible]ter; and combinations thereof.

2. The transgenic mammal of claim 1 [illegible]essing any of the constructs

3. Cells cultured from the transge[illegible]ammal of claim 1.

combination genomic-cDNA APP gene construct, with the addition of a mutation at amino acid 717, operably linked to a promoter selected from the following: the human APP promoter, mouse APP promoter, rat APP promoter, metallothionine promoter, rat neuron specific enolase promoter, human ß actin gene promoter, human platelet derived growth factor B (PDGF-B) chain gene promoter, rat sodium channel gene promoter, mouse myelin basic protein gene promoter, human copper-zinc superoxide dismutase gene promoter, and mammalian POU-domain regulatory gene promoter; and combinations thereof.

10. The method of claim 9 wherein the transgenic model is a mammal having altered behavior.

11. A method for screening compounds for an effect on Alzheimer's disease comprising

exposing a transgenic mammal or mammalian cells containing a construct selected from the group consisting of the APP770 cDNA; the APP770 cDNA bearing a mutation at amino acid 717; the APP751 cDNA containing the KI protease inhibitor domain without the OX2 domain in the construct; the APP751 cDNA bearing a mutation at amino acid 717; the APP695 cDNA; the APP695 cDNA bearing a mutation at amino acid 717; the APP leader sequence followed by the ß peptide region plus the remaining carboxy terminal 56 amino acids of APP; the APP leader sequence followed by the ß peptide region plus the remaining carboxy terminal 56 amino acids with the addition of a mutation at amino acid 717; APP leader sequence followed by the ß peptide region; the ß peptide region plus the remaining carboxy terminal 56 amino acids of APP; the ß peptide region plus the remaining carboxy terminal 56 amino acids of APP with the addition of a mutation at amino acid 717; a combination genomic-cDNA APP gene construct; a combination genomic-cDNA APP gene construct, with the addition of a mutation at amino

KI domain

B peptide

OX2 domain

Leader sequence

FIG. 1a

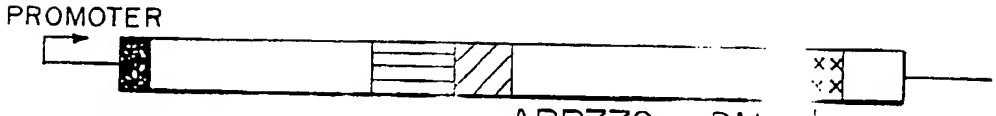


FIG. 1b

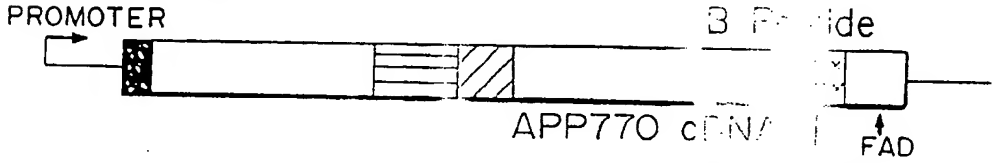


FIG. 2a

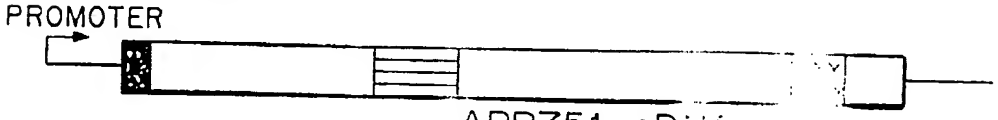


FIG. 2b

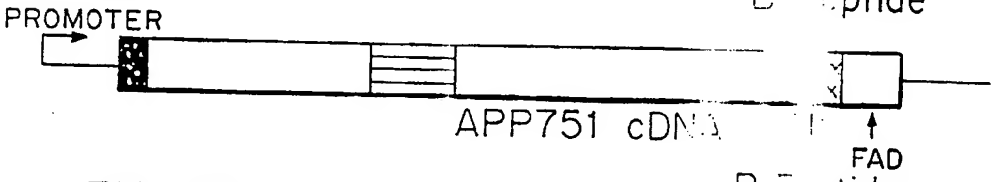
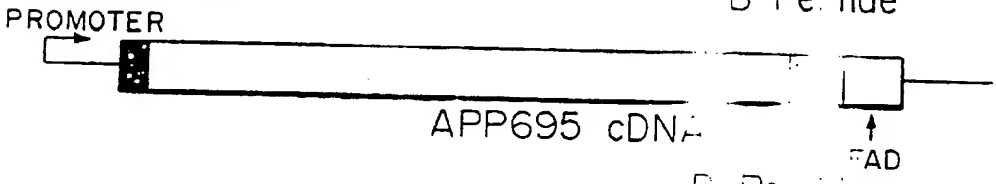


FIG. 3a

FIG. 3b



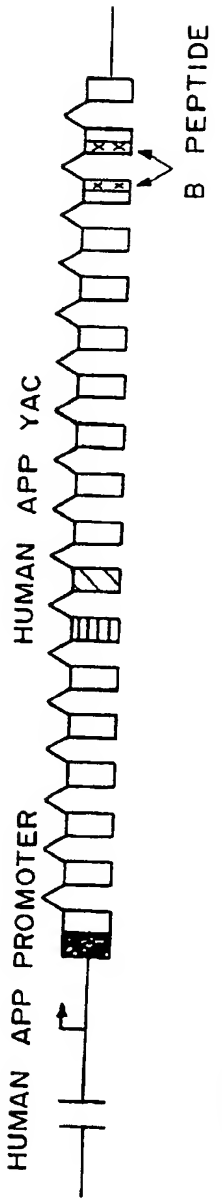


FIG. 7a

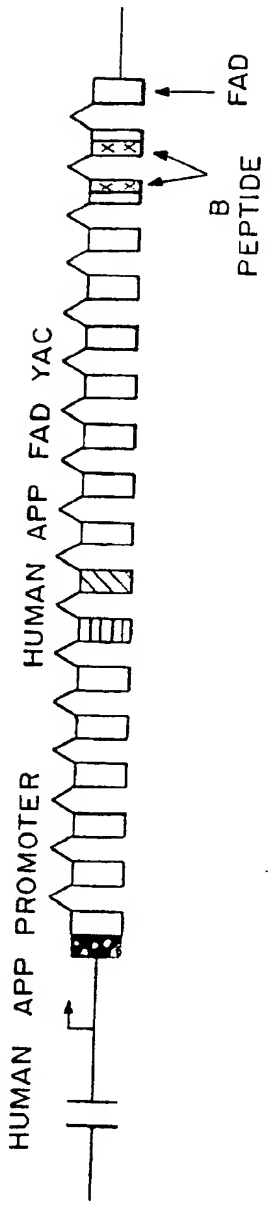


FIG. 7b

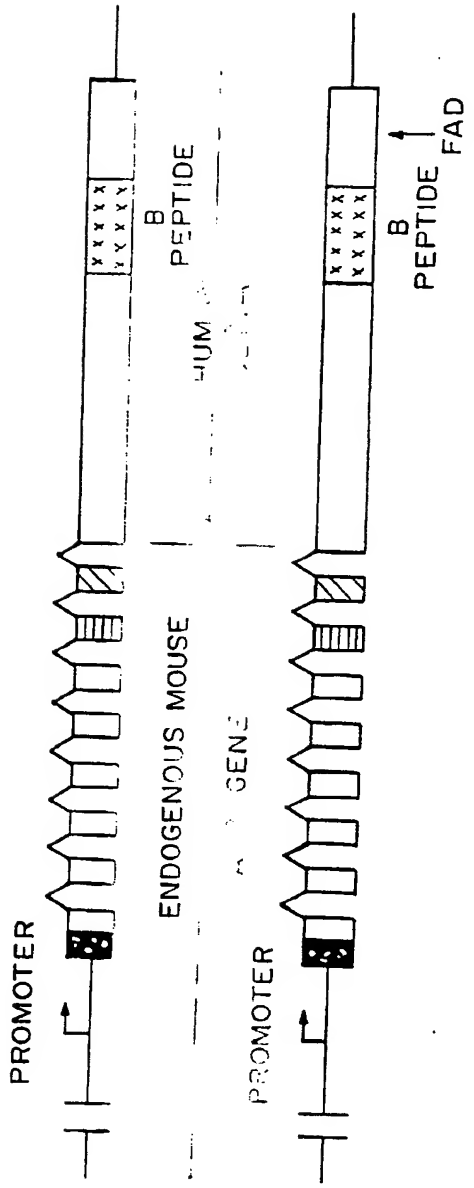


FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 92/11276

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Current opinion in neurology and neurosurgery, Volume 5, 1992, B. T. Hyman et al., "Amyloid, dementia and Alzheimer's disease", page 88 - page 93, fig 2, page 88, right column - page 89, left column, page 90, right column	1-13
Y	--	1-13
X	Journal of Biological Chemistry, Volume 266, No 32, November 1991, F. A. Sandhu et al., "Expression of the Human beta-Amyloid Protein of Alzheimer's Disease Specifically in the Brains of Transgenic Mice", pages 21331-21334, Fig 1, page 21334	1-6,9,10
Y	--	1-13
X	WO, A1, 8906689 (THE MCLEAN HOSPITAL CORPORATION), 27 July 1989 (27.07.89), page 21, line 18 - line 20; page 40 - page 46, figur 8, page 40, last paragraph	1,9
	--	
X	Nature, Volume 354, December 1991, S. Kawabata et al., "Amyloid plaques, neurofibrillary tangles and neuronal loss in brains of transgenic mice overexpressing a C-terminal fragment of human amyloid precursor protein", page 476 - page 478, figure 1, page 478	1-6,9-13
	--	
X	EP, A1, 0451700 (D. O. WIRAK), 16 October 1991 (16.10.91), page 6, line 35 - page 7, line 5; page 10 - page 13, examples 11-16	1-6,9-13
	--	
X	Science, Volume 253, July 1991, D. O. Wirak et al., "Deposits of Amyloid beta Protein in the Central Nervous System of Transgenic Mice", page 323 - page 325, page 323	1,9
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 92/11276

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

The present application lacks unity of invention a posteriori - see the cited documents. Thus, each of the claimed solutions in claims 1, 9 and 11 to the known problem of creating a transgenic animal modell for Alzheimers disease, represents a separate inventive concept.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

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SN	Senegal
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TD	Chad
TG	Togo
UA	Ukraine
US	United States of America
VN	Viet Nam

annual cost of $40 billion (Jorm, 1987; Fisher, LM: New York Times, August 23, 1989 D1 "Alzheimer's Disease", edited by Reisberg, B.; (The Free Press, New York & London 1983). These factors imply preventive action must be taken to decrease AD incidence by allocating resources into AD research.

At a macroscopic level, the brains of AD patients are usually smaller, sometimes weighing less than 1,000 grams. At a microscopic level, the histopathological symptoms of AD include neurofibrillary tangles (NFT), neuritic plaques, and degeneration of neurons. AD patients exhibit degeneration of nerve cells in the frontal and temporal cortex of the cerebral cortex, pyramidal neurons of hippocampus, neurons in the medial, medial central, and cortical nuclei of the amygdala, noradrenergic neurons in the locus coeruleus, and the neurons in the basal forebrain cholinergic system. Loss of neurons in the cholinergic system leads to a consistent deficit in cholinergic presynaptic markers in AD (Reisberg, 1983; "Alzheimer's Disease and related disorders, research and development" edited by Kelly WE; (Charles C. Thomas, Springfield, IL. 1984).

AD is associated with neuritic plaques measuring up to 200 μm in diameter in the cortex, hippocampus, subiculum, hippocampal gyrus, and amygdala. One of the principal constituents of neuritic plaques is amyloid, which is stained by congo red (Reisberg, 1983; Kelly, 1984). Amyloid plaques are extracellular, pink - or rust-colored in bright field, and birefringent in polarized light. The plaques are composed of polypeptide fibrils and are often present around blood vessels, reducing blood supply to various neurons in the brain.

Various factors such as genetic predisposition, infectious agents, toxins, metals, and head trauma have all been suggested as possible mechanisms of AD

cytoplasmic domain. The ß peptide consists of 28 amino acids just outside the membrane and 14 residues of the hydrophobic transmembrane domain. Thus, the ß peptide is a cleavage product of APP normally found in brain and other tissues such as heart, kidney and spleen. ß peptide deposits, however, are usually found only in the brain, although Joachim et al., Nature 341:226-228 (1989) have reported ß peptide deposits outside the brain in the skin, intestine, and subcutaneous tissues of most AD patients.

The larger alternate forms of APP (APP751, APP770) consist of all of APP695 plus one or two additional domains. APP751 consists of all of APP695 plus an additional 56 amino acids which has homology to the Kunitz family of serine protease inhibitors (KP1) (Tanzi et al., 1988; Weidemann, et al., Cell 57:115-126 (1989); Kitaguchi, et al., Nature 331:530-532 (1988); Tanzi et al., Nature 32[illegible]156 (1987). APP770 contains APP751 and an additional 19 amino acid domain homologous to the neuron cell surface antigen OX-2 (Weidemann, et al., Cell 57:115-126 (1989); Kitaguchi et al., 1988). APP is post-translationally modified by the removal of the leader sequence and by the addition of sulfate and sugar groups.

Van Broeckhaven, et al., Science 248:1120-1122 (1990) have demonstrated that the APP gene is tightly linked to hereditary cerebral hemorrhage with amyloidosis (HCHWA-D) in two Dutch families. This was confirmed by the finding of a point mutation in the APP coding region in two Dutch patients (Levy et al., Science 248:1124-1128 (1990). The mutation substituted a glutamine for glutamic acid at position 22 of the ß peptide (position 618 of APP695). In addition, certain families are genetically predisposed to Alzheimer's disease, a condition referred to as familial Alzheimer's disease (FAD), through mutations resulting in an amino acid replacement at position 717

and the protein coding sequence use For example, Wirak, et al. (1991) found that in ansgenic mice expressing a form of the ß peptide, ntracellular deposits of "amyloid-like" material reactive with antibodies prepared against APP wer observed but did not find other histopathological di ase symptoms. The intracellular nature of the ant ody-reactive material and the lack of other symp ms suggest that this particular transgenic animal i not a faithful model system for Alzheimer's diseas . Kawabata et al. (1991) report the production of amy id plaques, neurofibrillary tangles, and neuron cell death in their transgenic animals. In each these studies, the same peptide fragment, the ß pe ide plus the 56 remaining C terminal amino acids of PP, was expressed. Wirak et al. (1991) use the human APP promoter while Kawabata, et al. (19) used the human thy-1 promoter. In transgenic mice xpressing the APP751 cDNA from the neuron-specifi enolase promoter of Quon, D., et al., Nature 352, 23 241 (1991), extracellular deposits of material active with antibody prepared against APP were served. What was not shown was whether the deposits ntained full-length APP751 or ß peptide or both, hus precluding any correlation of the deposits wit those present in Alzheimer's disease. Quon et al. (91) also state that the protein encoded by the APP 5 cDNA expressed from the neuron-specific enolase pr oter, does not form extracellular immunoreactive d osits. These results raise the possibility that though the ß pept de is included within the APP6 precursor, use of the neuron-specific enolase prom er in conjunction with the APP695 cDNA may not presen an effective Alzheimer's disease model. Further re, the presence of APP immunoreactive deposits is n correlated with the age or gene dosage in their par cular transgenic model.

It is a still further object o the present invention to provide transgenic ani ls characterized by certain genetic abnormalities in he expression of the amyloid precursor protein.

Summary of the Inve on

The construction of transgenic nimal models for testing potential treatments for Al eimer's disease is described. The models are chara erized by a greater similarity to the condition existing in naturally occurring Alzheimer's dis se, based on the ability to control expression of on or more of the three forms of the ß-amyloid precur r protein (APP), APP695, APP751, and APP770, or subf gments thereof, as well as various point mutations sed on naturally occurring mutations, such as the FA mutations at amino acid 717, and predicted mutat ns in the APP gene. The APP gene constructs are epared using the naturally occurring APP promoter of uman, mouse, or rat origin, as well as inducible pr oters such as the mouse metallothionine promoter, whi can be regulated by addition of heavy metals such as inc to the animal's water or diet. Neuron-spe fic expression of constructs is achieved by using the at neuron specific enolase promoter.

The constructs are introduced to animal embryos using standard techniques such as m roinjection or embryonic stem cells. Cell culture ased models can also be prepared by two methods. C l cultures can be isolated from the transgenic animal or prepared from established cell cultures using the ame constructs with standard cell transfection tec niques.

The specific constructs that a described employ the following protein coding sequen s: the APP770 cDNA; the APP770 CDNA bearing a mut ion at amino acid 717; the APP751 cDNA containing th KI protease inhibitor domain without the OX2 do ain in the

replaced by the analogous human seq nces; the endogenous mouse or rat APP gene mo fied through the process of homologous recombination etween the APP gene in a mouse or rat ES cell and vector carrying the human APP cDNA bearing a mutati at amino acid position 717 such that sequences in he resident rodent chromosomal APP gene beyond e recombination point (the preferred site for recom nation is within APP exon 9) are replaced by the ana gous human sequences bearing a mutation at ami acid 717. These constructs can be introduced into t transgenic animals and then combined by mating f animals expressing the different constructs

The transgenic animals, or ani l cells, are used to screen for compounds altering th pathological course of Alzheimer's Disease as me ured by their effect on the amount and histopatho gy of APP and ß peptide in the animals, as well as behavioral alterations.

Brief Description of the rawings

The boxed portions of the draw gs indicate the amino acid coding portions of the c structs. Filled portions indicate the various domai of the protein as indicated in the Figure Legend. ines indicate sequences in the clones that are 5' r 3' untranslated sequences, flanking genomic sequenc , or introns. The break in the line to the left o the constructs in Figs. 7 and 8 indicates the presenc of a long DNA sequence.

Figure 1a is a schematic of th APP 770 cDNA coding sequence.

Figure 1b is a schematic of th APP770 cDNA coding sequence bearing a mutation position 717.

Figure 2a is a schematic of th APP751 cDNA coding sequence.

Detailed Description of th· Invention

The constructs and transgenic imals and animal cells are prepared using the method and materials described below.

Sources of materials.

Restriction endonucleases are tained from conventional commercial sources suc as New England Biolabs (Beverly, MA.), Promega Bio gical Research Products (Madison, WI.), and Strata ne (LaJolla CA.), etc. Radioactive materials are obt ned from conventional commercial sources suc as Dupont/NEN or Amersham. Custom-designed oligonuc otides for site-directed mutagenesis are available om any of several commercial providers of such materi s such as Bio-Synthesis Inc., Lewisville, TX. Ki for carrying out site-directed mutagenesis are avail le from commercial suppliers such as Promeg Biological Research Products, Stratagene, etc. Clones of cDNA including the APP695, APP751, and A 770 forms of APP mRNA were obtained directly from Dr Dmitry Goldgaber, NIH. Libraries of DNA are availabl from commercial providers such as Stratagene, La Jo a, CA., or Clontech, Palo Alto, CA. PC12 and 3 cells were obtained from ATCC (#CRL1721 and #C 92 respectively). An additional PC12 cell line was ob ined from Dr. Charles Marotta of Harvard Medical hool, Massachusetts General Hospital, and cLean Hospital. Standard cell culture media appropr te to the cell line are obtained from conventional commercial sources such as Gibco/BRL. Murine stem cel , strain D3, were obtained from Dr. Rolf Kemler (Doet hman, et al., J. Embryol. Exp. Morphol. 87, 27 (1985 . Lipofectin for DNA transfection and the drug G418 or selection of stable transformants are available om Gibco/BRL.

exons in the APP gene (Lemaire et a ., Nucl. Acid Res, 17;517-522, 1989; Yoshikai et al., 90). These results taken together indicate tha the minimum size of the Alzheimer's gene is 175 kb.

I. Table of Alzheimer's Cosm and Lambda Clones

Library	Name of Clone	Insert Size (Kb)	signed APP Region
	1 GPAPP47A	35	5 Kb promoter & 9 Kb intron
1 Cosmid	2 GPAAP36A	35	2 Kb promoter & 22 Kb intron
1	3 GAPP30A	30-35	3' coding region
	4GAPP43A	30-35	exons 9, 10 and 11
	1 GAPP6A	12	exon 6
	2 GAPP6B	18	exons 4 and 5
	3 GAPP20A	20	exon 6
	4 GAPP20B	17	exons 4 and 5
Lambda	5 GAPP28A	18	exons 4 and 5
	6 GAPP3A	14	exon 6
	7 GAPP4A	19	exon 6
	8 GAPP10A	16	exons 9, 10 and 11
	9 GAPP16A	21	exon 6

Construction of Transgenes.

The clones bearing various po ions of the human APP gene sequence shown in Figs. 1- are constructed in an analogous manner. First, th polyA addition signal from SV40 virus as a 253 ba pair *Bcl*I to *Bam*HI fragment (Reddy et al., Scie 200;494-502 (1978) is cloned into a modified v tor from the pUC series. Next, the cDNA coding seq nces (770, 751, or 695) are inserted. Correct orient ion and content of the fragments inserted is determin through restriction endonuclease mapping a limited sequencing.

The clones bearing various ca oxy terminal portions of the human APP gene seq nce shown in Figs. 4 and 5 are constructed through se ral steps in

cDNA clone is converted to an *Xho*I s e by site-directed mutagenesis. Cleavage of t resulting plasmid with *Xho*I cuts at the new *Xh* site and a pre-existing site at 930, and releases t KI and OX-2 coding sequence.

The plasmid thus generated serv as the acceptor for the KI and OX-2 alternative spli ng cassette. The alternative splicing cassette is created through a series of cloning steps. First, the aqI site at position 860 (numbering system of Ka , et al., 1987) in the genomic clone containing exor and adjacent downstream intron is converted to ar hoI site by site-directed mutagenesis. Cleavage f the resulting plasmid with *Xho*I cuts at the new *Xh* site and an *Xho*I site within the adjacent intror This fragment is cloned into the *Xho*I site in a pl mid vector. Second, the genomic clone containing exon 9 and adjacent upstream intron is cleaved ith *Xho*I (position 930) and cloned into the X I site of a plasmid vector. These two junction con/intron fragments are released from their re pective plasmid backbones by cleavage with *Xho*I and ither *Bam*HI or *Bgl*II, and cloned into the *Xho*I site of a plasmid vector. The resulting *Xho*I fragment is cleaved with either *Bam*HI or *Bgl*II and the genomi 6.6 kb *Bam*HI segment (Kitaguchi et al., 1988) con aining the KI and OX-2 coding region along with their lanking intron sequences are inserted. After clea ge with *Xho*I, this DNA segment is inserted into th *Xho*I site of the modified APP770 cDNA constructed abo e. These cloning steps generate a combination cDNA/ge omic expression clone that allows cells in a transge ic animal to regulate the inclusion of the KI and OX-2 domains by a natural alternative splicing mechani m. An analogous gene bearing a mutation at amino aci 717 is constructed by using the mutated for of APP770 cDNA described above.

Other alternatives for use in c trolling human APP expression in neurons include th human ß actin gene promoter (Ray et al., Genes and evelopment 5:2265-2273 (1991)), the human plate t derived growth factor B (PDGF-B) chain gene promote (Sasahara et al., Cell 64:217-227 (1991)), the ra sodium channel gene promoter (Maue et al., Neuron 4 23-231 (1990)), the human copper-zinc superoxide dis tase gene promoter (Ceballos-Picot et al., Bra Res. 552:198-214 (1991)), and promoters for membe of the mammalian POU-domain regulatory gene family (Xi et al., Nature 340:35-42 (1989)). The U-domain is the region of similarity between the fou mammalian transcription factors Pit-1, Oct-1, t-2, and *unc*-86, and represents a portion of the DNA- inding domain. These promoters are known or believe to result in expression specifically within the r rons of transgenic animals.

Expression of human APP in non- uronal brain cells can be directed by the promote for mouse myelin basic protein (Readhead et al., Cell 8:703-712 (1987)).

Yeast Artificial Chromosomes.

The constructs shown in Figure are constructed as follows. Large segments of huma genomic DNA, when cloned into certain vectors, can be ropagated as autonomously-replicating units in th yeast cell. Such vector-borne segments are refe ed to as yeast artificial chromosomes (YAC; Burke al. Science 236, 806 (1987)). A human YAC library i commercially available (Clontech, Palo Alto, CA) ith an average insert size of 250,000 base pairs (nge of 180,000 to 500,000 base pairs). A YAC clone o the Alzheimer's gene can be directly isolated by sc ening the library with the human APP770 cDNA. The in usion of all of the essential gene regions in the c ne can be confirmed by PCR analysis.

vector is constructed from a human P cDNA (695 or 770 form), either wild-type or muta at amino acid 717. Cleavage of the recombinatior ector, for example, at the *Xho*I site within ex 9, promotes homologous recombination within the irectly adjacent sequences (Capecchi, 1989). The er genous APP gene resulting from this event is normal p to the point of recombination, within exon 9 in thi example, and consists of the human cDNA sequenc hereafter.

Mutant Forms of APP Proteins

Expression clones identical tc hese but bearing mutations at the amino acid 717 of e full length protein, the site of FAD mutations, re also constructed. Mutations at amino ac 717 are created by site-directed mutagenesis (Vinc , et al., 1989) and include mutations of the wild-t e val codon to one of the following codons; ile, , gly, tyr, leu, ala, pro, trp, met, ser, thr, asn, n. Mutations of val-717 to ile, phe, and gly, have en described (Goate et al., 1991; Murrell, et a 1991; Chartier-harlin et al., 1991). None of the naturally-occurring mutations are charged or lky amino acids. Therefore it is believed that repla ment of val-717 with the other amino acids listed also promote the FAD syndrome and have properties t are useful for animal AD models.

Preparation of Constructs for Tran ctions and Microinjections

DNA clones for microinjection e cleaved with appropriate enzymes, such as *Sal*1, t1, etc., and the DNA fragments electrophoresed on 1 garose gels in TBE buffer (Maniatis et al., 1989) The DNA bands are visualized by staining with ethidi bromide, excised, and placed in dialysis bags contai ng 0.3 M sodium acetate, pH 7.0. DNA is electroel ed into the dialysis bags, extracted with phen -chloroform (1:1), and precipitated by two volumes of thanol. The DNA

detail in Hogan et al. Manipulating he mouse embryo, Cold Spring Har or Laboratory, Cold pring harbor, NY (1986), the tea hings of which are corporated herein.

Transgenic Mice

Female mic six weeks of age a induced to superovulate wi a 5 IU injection .1 cc, ip) of pregnant mare serum gonadotropin (P G; Sigma) followed 48 hou s later by a 5 IU i ection (0.1 cc, ip) of human ch rionic gonadotropin hCG; Sigma). Females are pla ed with males immed tely after hCG injection. Twenty-one hours after h , the mated females are sacrificed by CO_2 asphyx tion or cervical dislocation and embryos are recover from excised oviducts and placed in Dulbecco's p sphate buffered saline with 0.5 bovine serum album (BSA; Sigma). Surrounding cumulus cells are remov with hyaluronidase (1 mg/ml). Pronuclea embryos are then washed and placed in Earle's balanc salt solution containing 0.5% BSA (EBSS) in a 37. C incubator with a humidified atmosphere at 5% CO_2, 9 air until the time of injection.

Randomly cycling adult female ce are paired with vasectomized males. Swiss Webs r or other comparable strains can be used for is purpose. Recipient females are mated at the me time as donor females. At the time of embryo tran er, the recipient females are anesthetized with an in peritoneal injection of 0.015 ml of 2.5% avert per gram of body weight. The oviducts are exposed by single midline dorsal incision. An incision is th made through the body wall directly over the oviduct The ovarian bursa is then torn with watchmakers orceps. Embryos to be transferred are placed in DPB and in the tip of a transfer pipet (about 10-12 em yos). The pipet tip is inserted into the infundibul and the embryos

Embryonic Stem (ES) Cell Methods:

Introduction of cDNA into ES cells:

Methods for the culturing of ES cells and the subsequent production of transgenic animals, the introduction of DNA into ES cells by a variety of methods such as electroporation, calcium phosphate/DNA precipitation, and direct injection are described in detail in Teratocarcinomas and embryonic stem cells, a practical approach, ed. E.J. Robertson, (IRL Press 1987), the teachings of which are incorporated herein. Selection of the desired clone of transgene-containing ES cells is accomplished through one of several means. In cases involving random gene integration, an APP clone is co-precipitated with a gene encoding neomycin resistance. Transfection is carried out by one of several methods described in detail in Lovell-Badge, Teratocarcinomas and embryonic stem cells, a practical approach, ed. E.J. Robertson, (IRL Press 1987) or in Potter et al Proc. Natl Acad. Sci. USA 81, 7161 (19). Calcium phosphate DNA precipitation, direct injection, and electroporation are the preferred methods. In these procedures, 0.5×10^6 ES cells are plated into tissue culture dishes and transfected with a mixture of the linearized APP clone and 1 mg of pSV2neo DNA (Southern and Berg, J. Mol. Appl. Gen. 1 327-341 (1982)) precipitated in the presence of 50 mg lipofectin in a final volume of 100 µl. The cells are fed with selection medium containing 10% fetal bovine serum in DMEM supplemented with G418 (between 200 and 500 µg/ml). Colonies of cells resistant to G418 are isolated using cloning rings and expanded. DNA is extracted from drug resistant clones and Southern blotting experiments using an APP770 cDNA probe are used to identify those clones carrying the APP sequences. In some experiments, PCR methods are used to identify the clones of interest.

Recipient fe.al s e mated such tha they will be at 2.5 to 3.5 d vs t-mating when req red for implantation it blastocysts contai ng ES cells. At the time of transfer, the rec ient females are anesthetized wi intraperitoneal njection of 0.015 ml of 2.5% tin per gram of ody weight. The ovaries are xp se by making an inc ion in the body wall directly o the oviduct and t ovary and uterus are e lized. A hole is de in the uterine horn 25 gauge needle rough which the blastocysts e nsferred. After e transfer, the ovary and uteru pushed back int the body and the incision is clo ed by two sutures. is procedure is repeated on he o site side if add ional transfers are to be ma e

Identificati f ransgenic Mice ar Rats.

Tail sa 1-2 cm) are remov from three week old animals. D prepared and ar yzed by both Southern blot a CR to detect tra genic founder (F_0) animals and he r rogeny (F_1 and F_2)

Patholo l udies

The var $_0$, F_1, and F_2 anima that carry the microinjecte a gene are sacrific l by CO_2 asphyxiation an alyzed by immunol tology for neuritic plaques d neurofibrillary angles (NFTs) in the brain. Bra of mice and rats om each transgenic l a fixed in 4% para ormaldehyde and sectioned on ostat. Sections e stained with antibodies r e with the APP an or the ß peptide. Secondary an bodies conjugated wit fluorescein, rhodamine, h adish peroxidase, alkaline phosphatase are ed to detect the imary antibody. These experime ermit identifica on of amyloid plaques and gionalization of ese plaques to specific are the brain. Plaqu ranging in size from 9 to 50 c aracteristically cur in the brains of AD patien in the cerebral cort , but also may be

The pr..e fr..ctions are dena ured in Laemmli sample buff..r ... electrophoresed c SDS-Polyacrylam .e s. The proteins :e be then transferred ..rocellulose filte ; by electroblott'.. The filters are b ocked, incubated with primary .. bo..ies, and finall reacted with enzyme conju..a.. s..condary antibod ..s. Subsequent incubation .ith. .he appropriate chr nogenic substrate reveals the po.. ion of APP protei ..

Pathol ic.. and Behavioral St lies

P.. .. ical Studies

Immunoh.. t.. egy and thioflavi.. S staining are conducted as d ..cribed elsewhere he ..ein.

In situ Hy ridizations: Radio ctive or enzymatical..y .. beled probes are us d to detect mRNA *in situ*. T.. pr..bes are degraded a proximately to 100 nucleotides n .. .gth for better p..etration of cells. The procedu.. .. .hou et al. J. Psy h. Res. 24,27-50 (1990) for f.. .. and paraffin embe.. ed samples is briefly desc..i..d below although si ilar procedures can be emplo..e.. with samples sectio ed as frozen material. ..ara..fin slides for *in s tu* hybridization are dewaxed n .. .ene and rehydrate in a graded series of et..a.. .. and finally rins d in phosphate buffered sali.. (PBS). The sectio.. are post-fixed in fresh 4% paraf.. maldehyde. The sl.. es are washed with PBS twice fo.. .. minutes to remove .. raformaldehyde. Then the sec..ions are permeabilize by treatment with a 20 µg/ml .. ot..inase K solution. .he sections are re-fixed in .% p.. aformaldehyde, a .. basic molecules that could g..ve .. se to background robe binding are acetylated in a 0.1 M triethanolam.. e, 0.3 M acetic anhydride solu.. on for 10 minutes. The slides are washed in PB.., .hen dehydrated in a graded series of ethanols and a.. dried. Sections .. e hybridized with antisense p..b.., using sense probe s a control. After approp..i..t.. washing, bound r.. ioactive probes

NIH3T3 or P[illegible] were plated in [illegible] 100 mm dishes and transfected with a mixture of 5 [illegible]g of the *Sal*1 fragment and 1 [illegible] of pSV2neo DNA (4[illegible] precipitated in the presence of [illegible] mg lipofectin (G[illegible]co, BRL) in a final volume of 100 µl. Polylysine [illegible]oated plates were used for PC1 [illegible]ells, which normally [illegible]o not adhere well to tissue cu[illegible]e [illegible]ishes. The cell[illegible] were fed with selection medi[illegible]ntaining 10% fet[illegible] bovine serum in DMEM or RPMI a[illegible]pplemented with [illegible]418. Five hundred mg/ml (biolo[illegible] weight) and 250 m[illegible]/ml of G418 were used to sele[illegible] co[illegible]nies form NIH3T3 [illegible]nd PC12 cells, respectively. Fifteen days after t[illegible]nsfection, colonies of [illegible]ls resistant to G418 [illegible]ere isolated by cloning ring[illegible]a expanded in T fla[illegible]s. The presence of APP cDNA [illegible] cells was detect[illegible] by PCR using the procedure of [illegible] and Faloona, Me[illegible]ods Enzymol. 155;335-350 [illegible]87), the teachings o[illegible] which are incorporated herein.

Expression of APP in 25 coloni[illegible]s from each cell line was ana[illegible]ed by immunostaining (Majocha et al., 1988). Cell[illegible] wer[illegible] grown to subconf[illegible]ence and fixed in a solution co[illegible]ining 4% paraformal[illegible]ehyde, 0.12 M NaCl, and 20 [illegible] $NaPO_4$, pH 7.0. The [illegible] were incubated overnight wi[illegible] a primary monoclonal [illegible]antibody against a synthetic ß [illegible]eptide sequence (Maste[illegible]s et al., 1985) provided by [illegible] Ronald Majocha, Mas[illegible]achusetts General Hospital, Bo[illegible]ton, MA, followed by a [illegible]generalized anti-mouse antibody [illegible]jugated to biotin (Jackson ImmunoResearch [illegible]s, PA). Immunost[illegible]ining was then performed by [illegible]ding avidin-horserad[illegible]sh peroxidase (HRP) (Vecto[illegible] Labs, Burlingame, CA) and diaminobenzi[illegible]ine as the chromogen ([illegible]ajocha et al., 1985). The [illegible]sults indicated that [illegible]he pMTAPP-1 vector was expressing APP in both NIH3T3 a[illegible]d PC12 cells.

Example 2: [illegible]xpression of pEAPP-[illegible] in PC12 Cells.

pEAPP-1 [illegible] an example of the [illegible]2 kb human APP gene promoter li[illegible]e[illegible] to and controlling [illegible]xpression of a

thus encoding the leader sequence, followed by a shortened portion of the ß peptide starting with the sequence asp-val-gly-ser-of the ß peptide followed by the 5 terminal amino acids of APP. DNA from this construct was transfected into PC12 cells as described above.

Example 3 Generation of Transgenic Mice Expressing APP under the control of the MT-1 promoter.

Transgenic mice were made by microinjecting pMTAPP1 DNA into pronuclear embryos. pMTAPP1 is an example of the type of construct shown in Fig. 1a which is operably linked to the metallothionine promoter. The procedures for microinjection into mouse embryos are described in "Manipulating the mouse embryo" by Hogan et al. (1986). Only a brief description of the procedures is described below.

Mice were obtained from Taconic Laboratories (German Town, New York). Swiss Webster female mice were used for embryo retrieval and implantation. $B6D2F_1$ males were used for mating and vasectomized Swiss webster studs were used to simulate pseudopregnancy.

Embryo recovery: Female mice, 4 to 8 weeks of age, were induced to superovulate with 5 IU of pregnant mare's serum gonadotropin (PMSG; Sigma) followed 48 hours later by 5 IU of human chorionic gonadotropin (hCG; Sigma). Females were placed with males immediately after hCG injection. Embryos were recovered from excised oviducts of mated females 21 hours after hCG in Dulbecco's phosphate buffered saline with 0.5% bovine serum albumin (BSA; Sigma). Surrounding cumulus cells were removed with hyaluronidase (0.1 mg/ml). Pronuclear embryos were then washed and placed in Earle's balanced salt solution containing 0.5% BSA (EBSS) in a 37.0°C incubator with

infundi.. and embryos were transferred. After the transfe... incision was closed by two sutures.

An... y ...f Mice For Trans...ene Integration: At three we... age tail samples abo...t 1 cm long were excised fo... A analysis. The tail samples were digeste... b... cubating with sha...ing overnight at 55°C in the pre... ce of 0.7 ml 5 mM tris, pH 8.0, 100 mM EDTA, 0.5... S and 350 µg of protei...ase K. The digeste... m... rial was extracted onc... with an equal volume o... ...ol and once with an e...ual volume of phenol:ch... ...orm (1:1 mixture). T...e supernatants were mix... ...h 70 µl 3 M sodiu... ac...tate (pH 6.0) and the DNAs w... precipitated by a...din... equal volume of 100% ethan... The DNAs were spun d...wn in a microfuge, washed onc... ith 70% ethanol, dried and dissolved in 100 µl TE ... fer (10 mM tris pH 8.0 and 1 mM EDTA).

10-2... of DNAs were restrict...d with *Bam*H1, electroph... ...d on 1% agarose g...ls, blotted onto nitrocell... ...e paper, and hybridize... with ^{32}P-labeled APP cDNA ... ...ment. Transgenic ...nim...ls were identified by autora... ...raphy of the hybri...ize... nitrocellulose filters. ... DNAs were also analyz...d by PCR carried out by a... ...ic primers to generat... an 800 bp fragment.

A to ... ...f 671 pronuclear ...mbr...os were microinje... out of which 73 live ...nd 6 dead pups were born ...NA analysis identified 9 transgenic mice (5 females ...d 4 males) which were ...red to generate F_1 and F_2 tran...enics. These animals can be analyzed for expressio... mRNA and protein of A...P in different tissues a... ...r analysis of behavio...al and pathologi... abnormalities as descr...bed above.

4. a.. al of claim 1 .rod ced by introduct of ne construct i.to .n embryo, insertion the mbryo into a surr gate mother, and allowing embryo to develop o t rm.

5. mammal of claim 1 wher.in the mammal is a rodents.

6. ma.al of claim 1 rod ced by mating transgeni mma.s expressing diffe.ent constructs.

7. mam.al of claim 1 wher.in the codon encoding val amino acid res.due at 717 in the wild type APP .e is mutated to encode .n amino acid selected the group consisting of ile, phe, gly, tyr, leu, , pro, trp, met, s.r, hr, asn, and gln.

8. mam.al of any of claim 1 wherein the promoter he APP promoter of the same species of origin as .e mammal.

9. ethod for making a tran genic model for Alzheime' isease comprising intr ducing into non-human mam.. an cells or embryo a construct selected from the p consisting of the AP770 cDNA; the APP770 cD. bearing a mutation at amino acid 717; the APP751 cD A containing the KI protease inhibitor domain wi.. ut the OX2 domain in the construct; the APP751 cL. bearing a mutation at amino acid 717; the APP695 cDI the APP695 cDNA bearing a mutation at amino aci 7; the APP leader sequence followed by the ß pep.. region plus the remaining carboxy terminal amino acids of APP; the APP leader sequence .o lowed by the ß peptide region plus the remaining .rboxy terminal 56 amino acids with the addition c mutation at amino acid 717; APP leader sequence .. owed by the ß peptide region; the ß peptide r gi.n plus the remaining carboxy terminal 56 amino aci of APP; the ß peptide region plus the remaining rboxy terminal 56 amino acids of APP with the addit.. of a mutation at amino acid 717; a combinati . enomic-cDNA APP gene construct; a

acid 717, ... ably linked to a ...rom ter selected from the fol...: the human APP pr...not r, mouse APP promote..., ...PP promoter, met...llo hionine promoter, rat neuron ...ific enolase pro...ote , human ß actin gene pr...mo... human platelet d...riv d growth factor B (PDGF-b, ... gene promoter, r...t s dium channel gene promoter, ... myelin basic pr...tei. gene promoter, human cop... ...nc superoxide dis...mut se gene promoter, and mamma... OU-domain regula...ory gene promoter; transge...ic ...ls or animal ce...ls ontaining a construct s... e...ted from the gro...p c...nsisting of a human y...as... ...ificial chromoso...e c...nstruct controlled by the hu...n ...P promoter and a hum...n yeast artificial chromosom... ...struct controlled by he human APP promoter ... ...e addition of a mut...tion at amino acid 717, and t... ...s...enic animals or ...nim...l cells containing an APP gen... ...e...ected from the g...oup consisting of the endogenous ... ...e or rat APP gen... mo...ified such that sequences ....e resident chrom...som...l APP gene beyond the recomb...tion point in APP exon 9 are replaced by the analo... ...human sequences a...d t...e analogous human sequences ... ...the addition of ... mu...ation at amino acid 717; a...d ...ombinations ther...of, to the compound to be tested ...d ...etermining if th...re ...s altered expressio... ... APP.

12. ... method of claim 11 us...ing transgenic mammals f... ...r comprising dete...mi...ing if there is altered b... ...r of the transgenic ...ammals after administr...t... of the compound o...the animal.

13. ... ...ethod of claim 1 wh...erein the mammals are roden... .

FIG. 4a

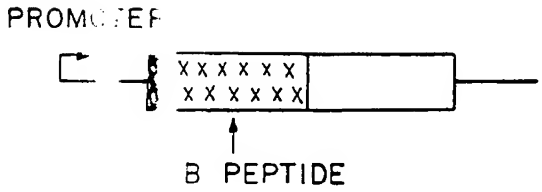


FIG. 4b

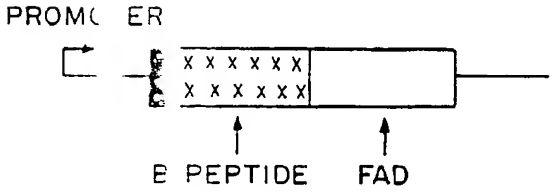


FIG. 5

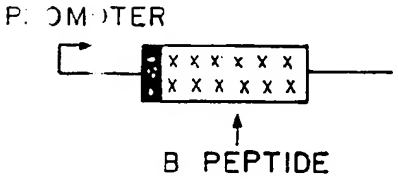


FIG. 6a

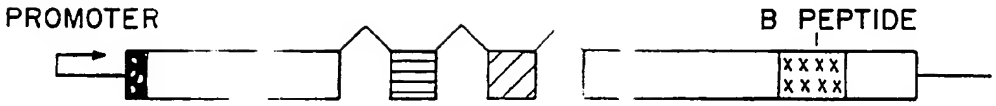
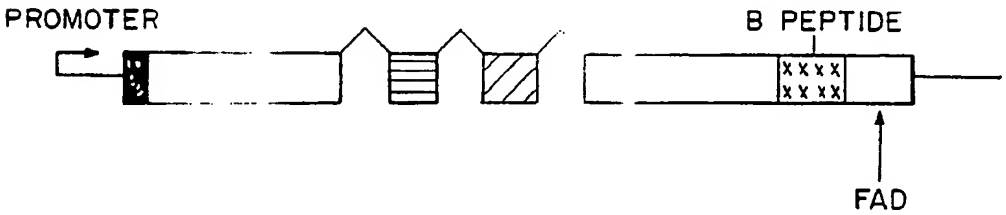


FIG. 6b



INTE[illegible] SEARCH REPORT

ternational application No.
PCT/US 92/11276

A. CLASSIFICATIO[illegible] T MATTER

IPC5: C12N 15/[illegible] 027, C12N 5/10, C07K 15/00
According to Internatio[illegible] atien (IPC) or to both national classification and IPC

B. FIELDS SEARCH[illegible]

Minimum documentation [illegible]ication system followed by classification symbols)

IPC5: C12N, C07[illegible]

Documentation searched o[illegible] mum documentation to the extent that such documents are included in the fields searched

Electronic data base cons[illegible] ernational search (name of data base and, where practicable, search terms used)

C. DOCUMENTS C[illegible] TO BE RELEVANT

Category*	Citation of [illegible] with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A1, [illegible] (CALIFORNIA BIOTECHNOLOGY INC.), 26 [illegible] 1991 (26.12.91), figures 4-7, claims 1-4 [illegible] 1-13, 15-16, 29-33, 61 and 64-65, exa[illegible] es [illegible], page 60, line 10 - page 61	1-6,9-13
Y	--	1-13
Y	Nature, [illegible] lume 352, July 1991, D. Quon et al., "For[illegible] of beta-amyloid protein deposits in brai[illegible] [illegible] nsgenic mice", page 239 - page 241, page[illegible] 39, [illegible] ge 241 left column	1-13
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[X] Further documents [illegible] listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
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"&" document member of the same patent family

Date of the actual comp[illegible]ion of [illegible] international search	Date of mailing of the international search report
19 April 1993	13. 05. 93
Name and mailing addres[illegible] of the [illegible] European Patent Office, [illegible]. 5818 Patentlaan 2 NL-2280 HV [illegible] wijk Tel. (+31-70) 3[illegible] 2040 [illegible] Tx. 31 651 epo nl, Fax (+31-70) 3[illegible]	Authorized officer Carl Olof Gustafsson

Form PCT/ISA/210 (second sh[illegible]) (J[illegible], 1992)

INTE[illegible]SEARCH REPORT

International application No.
PCT/US 92/11276

C (Continuation). DOC[illegible]ENTS CONSIDERED TO BE RELEVANT

Category*	Citation of [illegible] with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	The EM[illegible]nal Volume 10, No 2, 1991, D. [illegible]ak [illegible] al., "Regulatory region of human amy[illegible] precursor protein (APP) gene promotes neuro[illegible]pecific gene expression in the CNS of trans[illegible]ic mice", page 289 - page 296, figure 2, Discu[illegible]on	1-13
Y	Nature, [illegible]ume [illegible], February 1991, A. Goate et al. "Segregation of a missense mutation in the amy[illegible] precursor protein gene with familial Alzhei[illegible]r's disease" page 704 - page 706	1,7,9,11
Y	Nature, V[illegible]ume [illegible], October 1991, M-C. [illegible]rtie[illegible]arlin et al., "Early-onset alzheimer's disease caused by mutations at codon 717 [illegible] the beta-amyloid precursor protein gene" pag[illegible]44 - page 846	1,7,9,11
Y	Scienc[illegible], [illegible]ume 254, October 1991, J. Murrell et al., "A Mutation in the Amyloid Precursor Protein Associ[illegible]d [illegible] Hereditary Alzheimer's Disease"	1,7,9,11
P,X	WO, A, 92[illegible]187 (THE UPJOHN COMPANY), 16 April 1992 (16.0[illegible]2), page 8, line 15 - line 33, examples 1 and 2	1-6,8-13
P,X	WO, A, 921[illegible]09 (IMPERIAL COLLEGE OF SCIENCE, TECHNOLOGY & MEDICINE), 6 August 1992 (06.08.92), claims, examples 3 and 4, pages 10, 11, 13, 14 and 19-21	1-13
E,X	WO, A1, 9[illegible]189 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA), 4 February 1993 (04.02.93), claims, example	1-5,9,11-13

S. .9144

INTERNATIONAL SEARCH REPORT
Information on patent family members

31/03/93

International application No.
PCT/US 92/11276

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 911[illegible]1	26/12/91	AU-A- 8210291	07/01/92
WO-A1- 890668[illegible]	27/07/89	AU-A- 3056289	11/08/89
EP-A1- 04517[illegible]	16/10/91	NONE	
WO-A- 92061[illegible]7	16/04/92	AU-A- 8768191	28/04/92
WO-A- 921300[illegible]	06/08/92	AU-A- 1169492	27/08/92
WO-A1- 930218[illegible]	04/02/93	NONE	

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